A mouse model for *EML4-ALK*-positive lung cancer

Manabu Soda[a,b], Shuji Takada[a], Kengo Takeuchi[c], Young Lim Choi[a], Munehiro Enomoto[a], Toshihide Ueno[a], Hidenori Haruta[a], Toru Hamada[a], Yoshihiro Yamashita[a], Yuichi Ishikawa[c], Yukihiko Sugiyama[b], and Hiroyuki Mano[a,d,1]

Divisions of [a]Functional Genomics and [b]Pulmonary Medicine, Jichi Medical University, Tochigi 329-0498, Japan; [c]Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan; and [d]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan

Edited by John D. Minna, University of Texas Southwestern Medical Center, Dallas, TX, and accepted by the Editorial Board October 17, 2008 (received for review June 2, 2008)

EML4-ALK is a fusion-type protein tyrosine kinase that is generated in human non-small-cell lung cancer (NSCLC) as a result of a recurrent chromosome inversion, inv (2)(p21p23). Although mouse 3T3 fibroblasts expressing human EML4-ALK form transformed foci in culture and s.c. tumors in nude mice, it has remained unclear whether this fusion protein plays an essential role in the carcinogenesis of NSCLC. To address this issue, we have now established transgenic mouse lines that express EML4-ALK specifically in lung alveolar epithelial cells. All of the transgenic mice examined developed hundreds of adenocarcinoma nodules in both lungs within a few weeks after birth, confirming the potent oncogenic activity of the fusion kinase. Although such tumors underwent progressive enlargement in control animals, oral administration of a small-molecule inhibitor of the kinase activity of ALK resulted in their rapid disappearance. Similarly, whereas i.v. injection of 3T3 cells expressing EML4-ALK induced lethal respiratory failure in recipient nude mice, administration of the ALK inhibitor effectively cleared the tumor burden and improved the survival of such animals. These data together reinforce the pivotal role of EML4-ALK in the pathogenesis of NSCLC in humans, and they provide experimental support for the treatment of this intractable cancer with ALK inhibitors.

transgenic mouse | surfactant protein C | molecular targeted therapy

Lung cancer remains the leading cause of cancer deaths, with almost 1.3 million people dying annually from this condition worldwide (www.who.int/cancer/en). Although a variety of chemotherapeutic regimens have been developed to treat this intractable disease, their efficacy is limited and depends on cancer subtype. Non-small-cell lung cancer (NSCLC) accounts for 80–85% of all lung cancer cases and is less sensitive to cytotoxic drugs than is small cell lung cancer. Unless tumor cells are surgically resected at an early clinical stage, individuals with NSCLC can expect a median survival time of less than 1 year (1).

A subset of individuals with NSCLC (mostly nonsmokers, young females, and those of Asian ethnicity) have been shown to harbor mutations in the epidermal growth factor receptor (EGFR) gene (2–4). Such mutations result in constitutive activation of the EGFR tyrosine kinase, the oncogenic potential of which has been demonstrated in a transgenic mouse system (5). Small-molecule drugs that specifically inhibit the catalytic activity of EGFR have been found to exhibit clinical efficacy in the treatment of NSCLC patients, especially in those with an activated EGFR (6, 7).

We recently developed a system for the construction of retroviral cDNA libraries from small quantities of clinical specimens (8–10), and we applied this technology to NSCLC to screen for oncogenes that might be potential drug targets (11). With the use of a focus-formation assay performed with mouse 3T3 fibroblasts, we identified a fusion-type oncogene, *EML4-ALK*, in an NSCLC specimen of a smoker (12). A small inversion within the short arm of chromosome 2 was found to result in the ligation of *EML4* and *ALK*, leading to the production of a fusion protein consisting of the amino-terminal portion of EML4 and the intracellular region of the protein tyrosine kinase ALK. The coiled-coil domain within this portion of EML4 mediates the constitutive dimerization and activation of EML4-ALK, which is responsible for the generation of transformed cell foci in culture and the formation by these cells of s.c. tumors in nude mice. Although the inv (2)(p21p23) rearrangement responsible for the fusion event occurs recurrently in NSCLC patients, it remains to be demonstrated that *EML4-ALK* plays an essential role in the carcinogenesis of NSCLC harboring the fusion gene.

To address this issue, we have now engineered the expression of *EML4-ALK* in lung epithelial cells of transgenic mice. The surfactant protein C gene (*SPC*) is specifically expressed in type 2 alveolar epithelial cells, and a fragment of its promoter has been used widely for establishment of mouse lines that express transgenes specifically in lung epithelial cells (13–15). We therefore generated independent mouse lines in which *EML4-ALK* expression is driven by the *SPC* promoter, and we found that all such mice develop hundreds of adenocarcinoma nodules in both lungs within only a few weeks after birth. Furthermore, inhibition of EML4-ALK activity with a small-molecule drug induced rapid death of the tumor cells.

Results

Generation of *EML4-ALK* Transgenic Mice. To generate mice with lung-specific expression of *EML4-ALK*, we ligated a fragment of the *SPC* promoter (≈3.7 kbp) to a cDNA for EML4-ALK variant 1 with an amino-terminal FLAG epitope tag (12). The cDNA was, in turn, attached to an RNA splicing cassette and a polyadenylation signal (Fig. 1*A*). The transgene construct (≈8.3 kbp) was then injected into pronuclear-stage embryos of C57BL/6J mice, and the resulting progeny were screened for the presence of the transgene by Southern blot analysis. Seven founder mice positive for incorporation of the transgene (copy number per diploid genome ranging from 1 to 30) were obtained (Fig. 1*B* and data not shown). Two transgenic lines (501-3 and 502-4, with 10 and 30 copies of the transgene per genome, respectively) were independently maintained by backcrossing to C57BL/6J mice. To confirm the lung-specific expression of the transgene, we performed RT-PCR analysis to detect *EML4-ALK* mRNA in an F_1 mouse of the 502-4 line. The transgene was expressed in lung tissue (containing adenocarcinoma nodules, see below) but not in liver, esophagus, stomach, colon, brain, kidney, or uterus (Fig. 1*C*).

Detection of Multiple Lung Adenocarcinoma Nodules in the Transgenic Mice. One founder mouse (503-6, with 3 copies of the transgene per genome) (Fig. 1*B*) died 3 weeks after birth. Postmortem

Author contributions: Y.I., Y.S., and H.M. designed research; M.S., S.T., K.T., Y.L.C., M.E., T.U., H.H., T.H., Y.Y., and Y.I. performed research; and H.M. wrote the paper.

Conflict of interest statement: K.T. is a consultant for Dako.

This article is a PNAS Direct Submission. J.D.M. is a guest editor invited by the Editorial Board.

[1]To whom correspondence should be addressed. E-mail: hmano@jichi.ac.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0805381105/DCSupplemental.

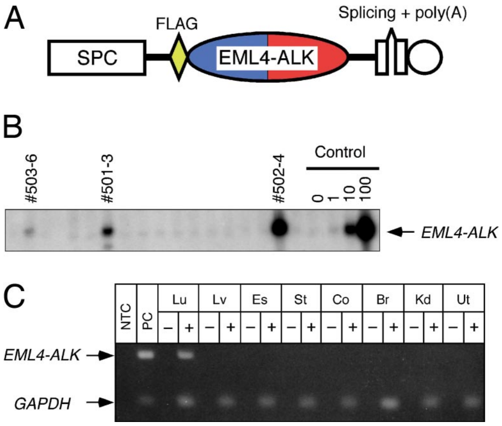

Fig. 1. Generation of transgenic mouse lines for *EML4-ALK*. (*A*) A cDNA for FLAG-tagged EML4-ALK was inserted between the *SPC* promoter and both splicing and polyadenylation [poly(A)] signal sequences. (*B*) Genomic DNA was isolated from the tail of founder mice generated from pronuclear-stage C57BL/6J embryos and was subjected to Southern blot analysis with full-length *EML4-ALK* cDNA as a probe. Control samples on the right comprised mouse genomic DNA with 0, 1, 10, or 100 copies of the transgene per diploid genome. The ID numbers of founder mice positive for the transgene are shown at the top. (*C*) Oligo(dT)-primed cDNA was synthesized from total RNA isolated from lung (Lu), liver (Lv), esophagus (Es), stomach (St), colon (Co), brain (Br), kidney (Kd), and uterus (Ut) of an F_1 mouse of the 502-4 line, with the reaction being performed in the presence (+) or absence (−) of reverse transcriptase. The cDNA preparations were then subjected to PCR with primer sets for *EML4-ALK* or for *GAPDH*, and the PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The positions of the PCR products are indicated on the left. RT-PCR was also performed for a no-template control (NTC) and for a human NSCLC specimen harboring *EML4-ALK* variant 1 as a positive control (PC).

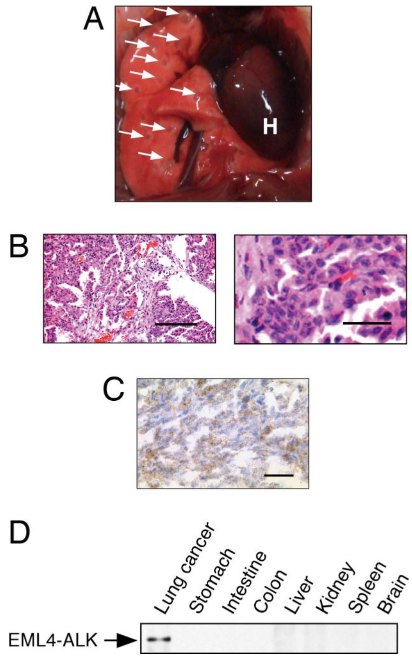

Fig. 2. Development of lung adenocarcinoma in *EML4-ALK* transgenic mice. (*A*) Hundreds of adenocarcinoma nodules (arrows) were apparent in the lungs of a founder mouse (503-6) that died 3 weeks after birth. H, heart. (*B*) Microscopic examination of the nodules shown in *A* after staining with H&E. Images at low (*Left*) and high (*Right*) magnification are shown with scale bars of 200 and 40 μm, respectively. Some tumors exhibited obvious scar formation, suggesting that they were invasive carcinomas. (*C*) Immunohistochemical analysis with antibodies to ALK of one of the nodules shown in *A* revealed a pattern of cytoplasmic staining with granular accentuations. (Scale bar, 50 μm.) (*D*) Immunoprecipitates prepared with antibodies to FLAG from the indicated tissues of an F_1 mouse of the 502-4 line were subjected to immunoblot analysis with the same antibodies. The position of EML4-ALK is shown at the left.

examination revealed hundreds of nodules in both lungs of this animal (Fig. 2*A*) and that these nodules were filled with adenocarcinoma cells (Fig. 2*B*). Immunohistochemical analysis with antibodies to ALK showed a diffuse cytoplasmic staining with granular accentuations in the neoplastic cells (Fig. 2*C*), consistent with the results of a similar analysis of *EML4-ALK*-positive human tumors (16). The level of immunoreactivity in the lungs of the transgenic mouse, however, was substantially lower than that in *EML4-ALK*-positive human specimens, suggestive of a lower level of expression for the EML4-ALK protein.

Detection of EML4-ALK by immunoblot analysis with antibodies to the FLAG tag confirmed a low-level but lung-specific expression of the kinase (Fig. 2*D*). Pathology and computed tomography (CT) examinations (see below) of the progeny of the maintained transgenic mouse lines (501-3 and 502-4) also revealed the development of multiple adenocarcinoma nodules in their lungs at only a few weeks after birth, demonstrating an essential role for the EML4-ALK kinase in NSCLC carcinogenesis. There was no discernable difference in tumor-forming activity between the 2 transgenic lines. We thus used both of these lines for further analyses.

Treatment of NSCLC-Positive Transgenic Mice with an ALK-Specific Inhibitor. To observe the development of NSCLC in the transgenic mice, we performed a series of CT scans of the chest. Multiple large nodules, some with infiltrative profiles of NSCLC, were detected in the lungs of progeny mice [Fig. 3*A*; also see supporting information (SI) Movie S1]. Other progeny with similar CT findings were subjected to pathology examination, confirming that such CT profiles reflected tumor expansion and infiltration in the lungs (data not shown). Examination of other organs of these mice failed to detect metastatic tumor nodules.

Several chemical compounds that specifically inhibit the tyrosine kinase activity of ALK have been identified (17–19). One such 2,4-pyrimidinediamine derivative has a median inhibitory concentration for ALK of <10 nM and a high specificity to ALK (Fig. S1) (20). We therefore examined whether peroral treatment with this compound (10 mg per kg of body weight per day) might inhibit the growth or induce the death of the adenocarcinoma cells in the transgenic mice. CT scans were performed after 0, 11, and 25 days of treatment for all 10 mice in each of the treatment and control (vehicle) groups, and a sequential examination of CT profiles was undertaken for each animal. The tumor mass developed rapidly in both lungs for most of the animals in the control group (Fig. 3*A*; also see Movie S2). Multiple nodules of various sizes newly appeared in the lungs, and the existing nodules became enlarged. In contrast, treatment with the ALK inhibitor greatly reduced the tumor burden in all mice (Fig. 3*B*). A large tumor in the lower lobe of the right lung in mouse 373, for instance, was reduced to ≈30% of its original size (based on the cross-section at the chest level in Fig. 3*B*) after only 11 days of the drug treatment and was almost undetectable by CT after treatment for 25 days (Movie S3). Sequential CT examination of another mouse (381) confirmed the pronounced activity of the ALK inhibitor (Fig. 3*B*; also see Movie S4 and Movie S5).

Mice in both groups were killed for pathology analysis after drug or vehicle administration for 2 months. Although multiple large tumor nodules were readily identified in the lungs of control mice, such nodules were apparent only occasionally in the treated animals (Fig. 3*C*), confirming the marked therapeutic effect of the ALK inhibitor. However, several small nodules were detected in the

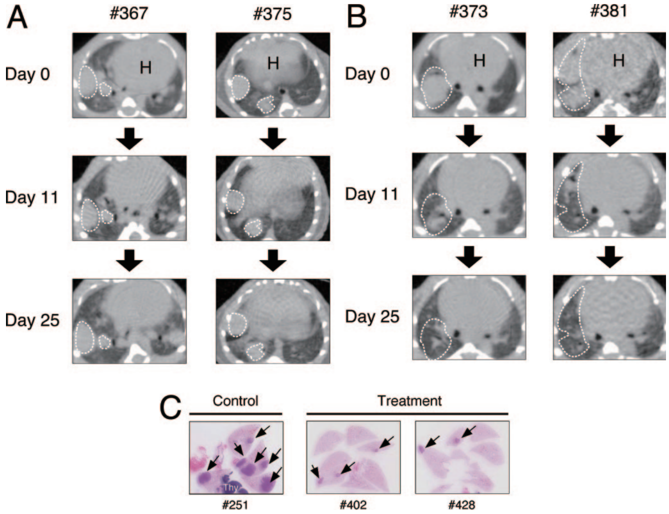

Fig. 3. Treatment of *EML4-ALK* transgenic mice with a specific ALK inhibitor. (*A* and *B*) Transgenic mice were subjected to daily peroral administration of vehicle (*A*) or ALK inhibitor (*B*) beginning at 4 weeks of age and were examined by CT scanning of the chest on days 0, 11, and 25. The ID numbers of the mice are shown at the top. H, heart. Tumors (indicated by broken lines) in both lungs underwent progressive enlargement in all control mice but became progressively smaller in all treated animals. (*C*) Macroscopic examination of the lungs from mice of the control and treatment groups at 2 months after the onset of treatment. The tissue was stained with H&E. The ID numbers of the mice are shown at the bottom. Cancer nodules are indicated by arrows. Thy, thymus.

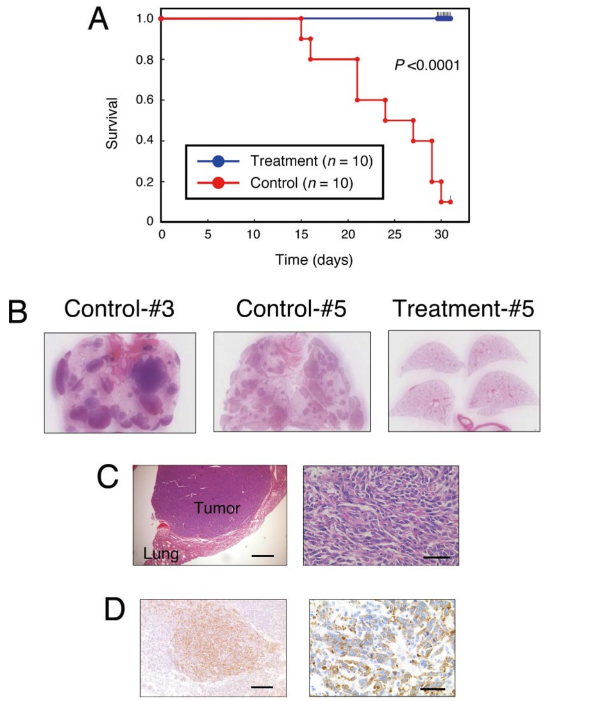

Fig. 4. Treatment with the ALK inhibitor of mice injected with EML4-ALK/3T3 cells. (*A*) Nude mice were injected i.v. with 2×10^5 3T3 cells expressing EML4-ALK variant 1 and were then immediately subjected to daily peroral administration of vehicle (control, $n = 10$) or ALK inhibitor (treatment, $n = 10$). Survival of the 2 cohorts is shown as a Kaplan–Meier plot and was compared by the log-rank test, with the calculated P value indicated. (*B*) Macroscopic examination of lungs isolated from mice of the control group at death or of the treatment group after treatment for 31 days. The tissue was stained with H&E. Most of the lungs in both control animals were occupied with transformed EML4-ALK/3T3 cells, whereas such cells were rarely observed in the treated animal. (*C*) Microscopic examination of lung tissue from a mouse of the control group after H&E staining. Images of low (*Left*) and high (*Right*) magnification are shown with scale bars of 500 and 50 μm, respectively. (*D*) Immunohistochemical analysis with antibodies to ALK of the nodules of EML4-ALK/3T3 cells that formed in the lungs of a mouse in the control group. Images of low (*Left*) and high (*Right*) magnification are shown with scale bars of 200 and 50 μm, respectively.

treated mice. Microscopic examination of the lungs of control and treated mice confirmed the changes observed by CT scanning and macroscopic analysis (data not shown). Even at this time point, we did not detect metastatic nodules in organs other than the lungs in either control or treated mice, and all animals in these cohorts survived the observation period.

Treatment of Mice Injected with 3T3 Cells Expressing EML4-ALK. Given that transgenic mice with lung cancer did not die by 6 months of age (with the exception of the one shown in Fig. 2*A* and another that died at 6 months after birth), we were not able to examine statistically the possible effect of the ALK inhibitor on survival in these animals. We therefore adopted another approach—that of loading mice with a large number of EML4-ALK-positive cells. We previously showed that mouse 3T3 fibroblasts expressing EML4-ALK (EML4-ALK/3T3) undergo transformation and generate s.c. tumors when injected into *nu/nu* mice (12). Such EML4-ALK/3T3 cells (2×10^5) were therefore injected i.v. into *nu/nu* mice ($n = 20$), and the ALK inhibitor was administered to half of these animals.

A total of 9 of the 10 untreated mice died within 1 month of injection with the EML4-ALK/3T3 cells (Fig. 4*A*). Postmortem examination of these mice revealed extensive dissemination of EML4-ALK-positive cells into the lungs (>60% of lung tissue was occupied with the transformed EML4-ALK/3T3 cells in all mice) (Fig. 4*B*). Pathology examination of the lungs revealed many nodules of various sizes that were filled with the EML4-ALK/3T3 fibroblasts (Fig. 4*C*). In a separate experiment, we confirmed that injection of parental 3T3 cells did not induce the formation of such nodules in the lungs or affect the survival of mice (data not shown).

To verify that the injected EML4-ALK/3T3 cells continued to express EML4-ALK, we stained tissue sections of the lungs of control mice with antibodies to ALK. All cells within nodules reacted with the antibodies (Fig. 4*D*), giving rise to a diffuse pattern of cytoplasmic staining with granular accentuations. Although the staining profile was similar to that observed for the transgenic mice, the staining intensity in the EML4-ALK/3T3 cell-injected animals was greater than that in the transgenic animals.

Similar to the results obtained with transgenic mice, transformed EML4-ALK/3T3 cells were not detected in any organs other than the lungs of the injected mice, with the exception of 2 animals in the control group (nos. 3 and 7). Given the massive infiltration of EML4-ALK/3T3 cells in the lungs of all mice in the control cohort, these mice likely died from respiratory failure. In the control no. 7 mouse, we detected pronounced infiltration of EML4-ALK/3T3 cells into both the mediastinum (Fig. S2*A*) and the diaphragm (Fig. S2*B*). Given that both of these structures are adjacent to the lungs and that this mouse had an exceptionally high tumor burden in the lungs (>90% of the lungs were occupied with EML4-ALK/3T3 cells; Fig. S2*C*), the presence of EML4-ALK/3T3 cells in the mediastinum and diaphragm was likely the result of direct invasion from the lungs rather than of distant metastasis.

Peroral administration of the ALK inhibitor markedly improved the outcome of mice injected with the transformed EML4-ALK/3T3 cells, with all 10 animals in the treatment group surviving the 1-month observation period ($P < 0.0001$, log-rank test) (Fig. 4*A*). The treated mice also were subjected to pathology analysis after this period, revealing the absence of EML4-

ALK/3T3 nodules from the lungs (Fig. 4*B*) and again demonstrating the high efficacy of the ALK inhibitor.

Discussion

We have shown here that the EML4-ALK fusion kinase plays an essential role in lung tumorigenesis. Hundreds of adenocarcinoma nodules developed simultaneously within a few weeks after birth in all independent lines of *EML4-ALK* transgenic mice examined. Given that the promoter fragment of *SPC* becomes active only at a late stage of gestation (21), a short period of *EML4-ALK* expression appears to be sufficient for full transformation. Although we did not examine *TP53* and *RB1* for possible abnormalities in the adenocarcinoma nodules of the transgenic mice, with both of these genes being frequently inactivated in human lung cancers (22), it is likely that only one (or at most a few) additional genetic event is required to generate cancer in EML4-ALK-expressing alveolar epithelial cells.

The expression level of EML4-ALK protein in the adenocarcinoma nodules of the transgenic mice was low. Given that the abundance of *EML4-ALK* mRNA in these nodules was found to be greater than that in human *EML4-ALK*-positive NSCLC specimens (data not shown), the expression of EML4-ALK protein appears to be suppressed in the mouse lung epithelial cells, possibly through translational or posttranslational mechanisms. The development of adenocarcinoma even at this low level of protein expression further reinforces the transforming activity of EML4-ALK.

Given the rapid development of NSCLC induced by EML4-ALK, the tumor cells are likely dependent for growth on the tyrosine kinase activity of the fusion protein. Such "oncogene addiction" (23) provides a potential target for the development of treatment strategies. We therefore tested whether inhibition of the enzymatic activity of EML4-ALK might reduce the tumor burden in the transgenic mice. The ALK inhibitor examined proved to be a promising candidate for the treatment of EML4-ALK-positive tumors. Furthermore, given the high sensitivity of the tumors in the transgenic mice to the ALK inhibitor, these animals provide a model system with which to examine the in vivo activity of other compounds or reagents targeted to ALK.

Many of the large tumors in the lungs of the transgenic mice changed to bullae or cysts after treatment with the ALK inhibitor, as revealed both by CT scanning (Fig. S3*A* and Movie S3 and Movie S5) and by pathology examination (Fig. S3*B*). Such a change was not described for treatment of activated EGFR-positive NSCLC in mouse models or humans with EGFR inhibitors (5, 6). A rapid induction of cell death by the ALK inhibitor in the transgenic mice may have triggered a collapse of the tumor burden within each nodule, thereby giving rise to bullae or cysts. Indeed, pathology examination revealed that a large tumor in 1 transgenic mouse (no. 250) became filled with necrotic tissue after treatment (Fig. S3*C*). However, the bullae cysts and necrotic tissue were still surrounded by remaining cancer cells (Fig. S3 *B* and *C*). Similarly, the lining tissue of some bullae cysts in the treated mice appeared to have a high density in CT scans (Fig. S3*A*), suggesting that peripheral cancer cells may survive in the nodules. Furthermore, small foci of cancer cells could be identified in the lungs of transgenic mice in the treatment cohort (Fig. 3*C*). Together, these various observations indicate that the current treatment protocol with the ALK inhibitor did not entirely eliminate tumor cells from the transgenic mice. Indeed, in a separate experiment transgenic mice treated with the 2,4-pyrimidinediamine for 25 days were examined 3 months after cessation of drug administration. Tumors of various sizes regrew in these mice (Fig. S4), indicative of the presence of surviving EML4-ALK-positive cancer cells in the animals after 25 days of drug treatment. Given that we have not tried other protocols or compounds, it remains unknown whether a total cure might be achieved by treatment for a longer period or with a higher dose of the same inhibitor or with a more potent compound. It is also possible that inhibition of additional signaling pathways, such as those mediated by phosphoinositide 3-kinase, mammalian target of rapamycin, or other protein tyrosine kinases (5, 24), may be required for a complete cure.

Despite the rapid growth of multiple tumors in the lungs of the transgenic mice, we failed to detect distant metastasis of such cancer cells in animals killed for analysis or in those that died within the total observation period of 6 months. However, we conclude that the tumors that developed in these mice had malignant characteristics on the basis of the following observations: (*i*) Histological analysis indicated that most tumors were noninvasive papillary adenocarcinomas, with some of them further showing obvious fibrosis and destruction of alveolar structures (Fig. 2*B*), a marker of invasion in human lung adenocarcinoma. (*ii*) Subcutaneous transplantation of tumor nodules that developed in the transgenic mice into the shoulder of *nu*/*nu* mice resulted in the growth of tumors at 6 of 8 injection sites in the recipient animals (Fig. S5*A*). (*iii*) Tumors that developed in the transgenic mice were shown to keep growing for at least 62 days in in vitro culture (Fig. S5*B*).

It is likely that expression of EML4-ALK (and probably other accompanying genetic changes) alone is not sufficient to render the cancer cells metastatic. It remains to be determined whether additional abnormalities in other oncogenes or tumor suppressor genes, such as *KRAS* or *LKB1* (25, 26), may lead to the generation of metastatic tumors in *EML4-ALK* transgenic mice.

Our present results have reinforced the importance of *EML4-ALK* in the pathogenesis of NSCLC in humans and have provided experimental support for the treatment of such intractable tumors with ALK inhibitors. Given that variants of *EML4-ALK* other than the variants 1 and 2 described in our original study (12) are now being identified (20, 27–29), it will be important to characterize all possible isoforms of *EML4-ALK* in humans to identify precisely the subgroup of patients who are candidates for future treatment with ALK inhibitors. Further to this goal, it will also be important to clarify the genetic changes that accompany the *EML4-ALK* fusion event as well as the downstream targets of EML4-ALK action in human NSCLC.

Materials and Methods

Generation of Transgenic Mice. A cDNA fragment encoding FLAG epitope-tagged EML4-ALK variant 1 (12) was ligated to the *SPC* promoter as well as to splicing and polyadenylation signals (Fig. 1*A*). The expression cassette was injected into pronuclear-stage embryos of C57BL/6J mice (PhoenixBio), and the copy number of the transgene was examined by Southern blot analysis with DNA from the tail of founder animals. All animal procedures were performed with the approval of the scientific committee for animal experiments of Jichi Medical University.

For detection of mRNAs derived from *EML4-ALK* and the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), total RNA was isolated from the organs of transgenic mice with the use of an RNeasy Mini kit (Qiagen) and was subjected to reverse transcription with SuperScript III reverse transcriptase (Invitrogen) and an oligo(dT) primer. Both reverse transcription and subsequent PCR analysis for each gene were performed as described previously (12).

For analysis of EML4-ALK protein in mice, organ homogenates were prepared with an Nonidet P-40 lysis buffer and subjected to immunoprecipitation with mouse monoclonal antibodies to FLAG (Millipore). The resulting precipitates were then subjected to immunoblot analysis with the same antibodies and a SuperSignal chemiluminescence kit (Pierce Biotechnology).

Pathology Examination. For immunohistochemical staining of EML4-ALK in EML4-ALK/3T3 cells, paraffin-embedded sections were depleted of paraffin with xylene, rehydrated with a graded series of ethanol solutions, and stained with mouse monoclonal antibodies to ALK (ALK1; Dako) at a dilution of 1:20 and with an EnVision+DAB system (Dako). The sections were subjected to heat-induced antigen retrieval with Target Retrieval Solution, pH 9.0 (Dako), before exposure to the antibodies. For detection of EML4-ALK in transgenic mice, cryostat sections were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min, treated with Target Retrieval Solution, pH 9.0, and immunostained with the monoclonal antibodies to ALK and the EnVision+DAB system.

Treatment with ALK Inhibitor. For the experiments based on i.v. administration of EML4-ALK/3T3 cells, the cells (2×10^5) were injected into the tail vein of 4-week-old *nu/nu* mice (Clea Japan). An inhibitor specific for the tyrosine kinase activity of ALK [example 3-39 in the patent application: Garcia-Echeverria C, *et al.*, inventors; Novartis AG, Novartis Pharma GmbH, IRM LLC, applicants (24 Feb 2005). 2,4-Pyrimidinediamines useful in the treatment of neoplastic disease and in inflammatory and immune system disorders. PCT WO 2005016894] was synthesized by Astellas Pharma and was orally administered each day at a dose of 10 mg/kg to the injected mice or to *EML4-ALK*-transgenic mice. Sequential examination of lung tumors was performed with an X-ray CT apparatus for experimental animals (LCT-100; Aloka).

ACKNOWLEDGMENTS. We thank Koichi Hagiwara (Saitama Medical University, Saitama, Japan) for kindly providing the human *SPC* promoter fragment as well as Keiko Shiozawa and Tomoyo Kakita for technical assistance. This study was supported in part by a grant for Research on Human Genome Tailor-made from the Ministry of Health, Labor, and Welfare of Japan; by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and by grants from the Japan Society for the Promotion of Science, the National Institute of Biomedical Innovation, the Princess Takamatsu Cancer Research Fund, the Takeda Science Foundation, the Uehara Memorial Foundation, the Smoking Research Foundation, the Vehicle Racing Commemorative Foundation, and the NOVARTIS Foundation (Japan) for the Promotion of Science.

1. Schiller JH, *et al.* (2002) Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 346:92–98.
2. Paez JG, *et al.* (2004) EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 304:1497–1500.
3. Pao W, *et al.* (2004) EGF receptor gene mutations are common in lung cancers from ''never smokers'' and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 101:13306–13311.
4. Shigematsu H, *et al.* (2005) Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 97:339–346.
5. Li D, *et al.* (2007) Bronchial and peripheral murine lung carcinomas induced by T790M-L858R mutant EGFR respond to HKI-272 and rapamycin combination therapy. *Cancer Cell* 12:81–93.
6. Lynch TJ, *et al.* (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350:2129–2139.
7. Kris MG, *et al.* (2003) Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: A randomized trial. *J Am Med Assoc* 290:2149–2158.
8. Hatanaka H, *et al.* (2007) Transforming activity of purinergic receptor P2Y, G-protein coupled, 2 revealed by retroviral expression screening. *Biochem Biophys Res Commun* 356:723–726.
9. Fujiwara S, *et al.* (2007) Transforming activity of purinergic receptor P2Y, G protein coupled, 8 revealed by retroviral expression screening. *Leuk Lymphoma* 48:978–986.
10. Choi YL, *et al.* (2007) Identification of a constitutively active mutant of JAK3 by retroviral expression screening. *Leuk Res* 31:203–209.
11. Besse B, Ropert S, Soria JC (2007) Targeted therapies in lung cancer. *Ann Oncol* 18(suppl 9):ix135–ix142.
12. Soda M, *et al.* (2007) Identification of the transforming *EML4-ALK* fusion gene in non-small-cell lung cancer. *Nature* 448:561–566.
13. Mishra A, Weaver TE, Beck DC, Rothenberg ME (2001) Interleukin-5-mediated allergic airway inflammation inhibits the human surfactant protein C promoter in transgenic mice. *J Biol Chem* 276:8453–8459.
14. Duan W, *et al.* (2002) Lung-specific expression of human mutant p53–273H is associated with a high frequency of lung adenocarcinoma in transgenic mice. *Oncogene* 21:7831–7838.
15. Zhao B, *et al.* (2000) Transgenic mouse models for lung cancer. *Exp Lung Res* 26:567–579.
16. Inamura K, *et al.* (2008) *EML4-ALK* fusion is linked to histological characteristics in a subset of lung cancers. *J Thorac Oncol* 3:13–17.
17. Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G (2008) The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nat Rev Cancer* 8:11–23.
18. McDermott U, *et al.* (2007) Identification of genotype-correlated sensitivity to selective kinase inhibitors by using high-throughput tumor cell line profiling. *Proc Natl Acad Sci USA* 104:19936–19941.
19. Galkin AV, *et al.* (2007) Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK. *Proc Natl Acad Sci USA* 104:270–275.
20. Choi YL, *et al.* (2008) Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. *Cancer Res* 68:4971–4976.
21. Korfhagen TR, *et al.* (1990) Cis-acting sequences from a human surfactant protein gene confer pulmonary-specific gene expression in transgenic mice. *Proc Natl Acad Sci USA* 87:6122–6126.
22. Testa JR, *et al.* (1997) Advances in the analysis of chromosome alterations in human lung carcinomas. *Cancer Genet Cytogenet* 95:20–32.
23. Sharma SV, Settleman J (2007) Oncogene addiction: Setting the stage for molecularly targeted cancer therapy. *Genes Dev* 21:3214–3231.
24. Engelman JA, *et al.* (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 316:1039–1043.
25. Ji H, *et al.* (2007) LKB1 modulates lung cancer differentiation and metastasis. *Nature* 448:807–810.
26. Huang CL, *et al.* (1998) Mutations of p53 and K-ras genes as prognostic factors for non-small cell lung cancer. *Int J Oncol* 12:553–563.
27. Rikova K, *et al.* (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 131:1190–1203.
28. Koivunen JP, *et al.* (2008) EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. *Clin Cancer* Res 14:4275–4283.
29. Takeuchi K, *et al.* (2008) Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res* 14:6618–6624.

MEDICAL SCIENCES